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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Byon et al.	Group Art Unit 1621
Appl. No.	:	09/970,043	
Filed	:	October 2, 2001	
For	:	**LOW MOLECULAR WEIGHT POLYMANNURONATE**	
Examiner	:	McIntosh III, Traviss C	

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on

2/13/03
(Date)

Mincheol Kim, Reg. No. 51,306

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Dr. Dong Soo Lee, do hereby declare as follows:

1. I received a Ph.D. in food chemistry from Pukyong National University at 599-1 Daeyun-3-Dong, Namgu, Busan, Korea in 1997, and now am working as the Head of Research Team in KBP Co, Ltd. at 584-1 Chilgoi-Dong, Pyongtaek-si, Gyeonggi-Do, Korea. A list of my representative publication is attached hereto as Appendix A.

2. As a co-inventor, I am familiar with the claimed invention of the above-identified patent application. I understand that in the August 13, 2002 Office Action, the Examiner rejected Claims 1-11, 13-29, 33-37, 51-61 relying on Eliaz et al. (U.S. Patent No. 6,274,566) in view of Dorian et al. (U.S. Patent No. 5,639,467), and Claims 41-50 relying on Eliaz et al. and Dorian et al. in further view of Iwata et al. (U.S. Patent No. 5,324,526). I have reviewed these references in preparing this Declaration.

3. In this declaration, I would like to set forth that the presently claimed polymannuronate with a molecular weight from about 40,000 Da to about 80,000 Da has very good cholesterol binding capability, which is substantially higher than polymannuronates with other molecular weight ranges.

4. In order to show the superior cholesterol controlling effects of the presently claimed polymannuronate over other materials, I conducted experiments comparing the cholesterol binding capability of polymannuronate of various molecular weights.

Preparation of Polymannuronates

5. I prepared 12 compositions of polymannuronate with various molecular weights according to the process described in Example 1.1 of the present specification. Initially, 12 samples, each of which contains 60g of alginate (average molecular weight of about 1300 kDa,) were prepared. Each sample was mixed with 30g of an aqueous solution of 0.4 M acetic acid. The 12 mixtures were stirred for hydrolysis at about 100°C for the different time periods as set forth in Table 1 below. Concentrated acetic acid was added to each of the hydrolysis resulting mixture to adjust its pH to 2.8-3.0. The pH-adjusted mixture was subjected to centrifugation. The supernatant was collected and neutralized by adding an aqueous solution of 1 M sodium carbonate. Then, ethanol was added to the neutralized solution to produce precipitation. The precipitated material was separated with the use of a centrifuge. The separated material was dissolved in distilled water. Ethanol was added to the aqueous solution to produce precipitation, which is separated by centrifugation to obtain substantially isolated polymannuronate.

Molecular Weights of Polymannuronates

6. The molecular weights of the obtained polymannuronates were measured using a Gel Permeation Chromatography (GPC), Model No. Waters 515, available from Waters Korea Ltd., using two columns, Ultra Hydrogel 500 and Ultra Hydrogel Linear, both available from the same company. The reflective index (RI) detector used was a Waters RI 410, also from Waters Korea Ltd. A solution of 0.1 N $NaNO_3$ was used as the eluent at a flow rate of 1 ml/min. During the operation, the columns were kept at 45 °C, and the RI detector was kept at 35 °C. The

measured molecular weights of the polymannuronates from the 12 samples are listed in Table 1 with the time periods for the hydrolysis.

Table 1

Sample No.	Time for Hydrolysis (min.)	Molecular Weight (Kda.)
1	20	210.3
2	25	155.5
3	30	122.1
4	40	92.6
5	45	82.9
6	50	72.3
7	55	62.8
8	60	53.2
9	150	44.2
10	240	32.4
11	360	23.0
12	480	10.2

Cholesterol Binding Capability of Polymannuronates

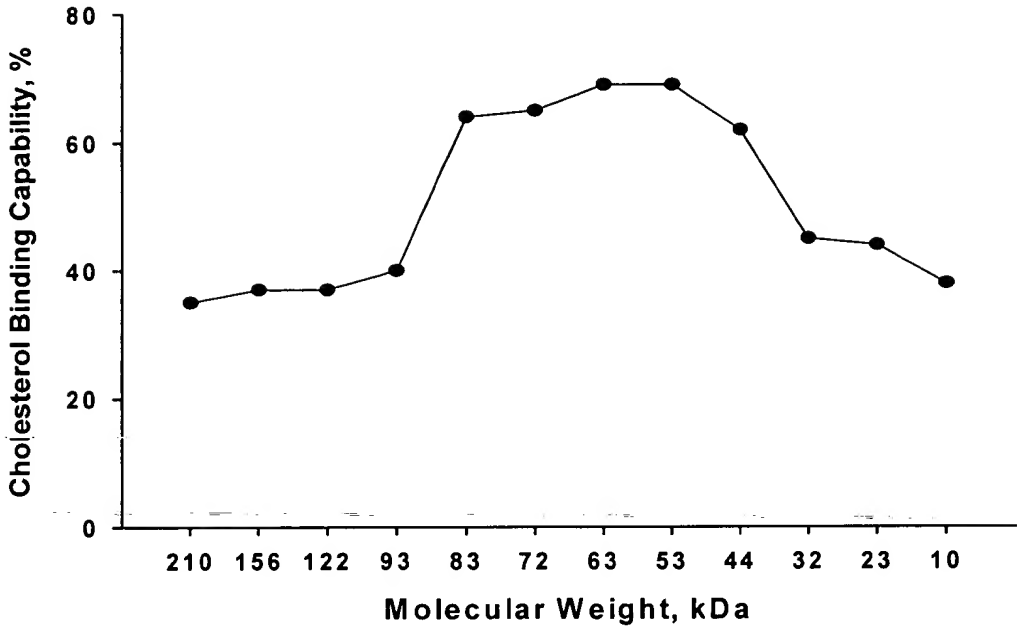
7. Binding capability of the polymannuronate with cholesterol was measured for each of the various molecular-weight polymannurontes. To 10 ml of 1 % (0.1 g) cholesterol solution in glacial acetic acid, 0.3 g of each polymannuronate sample was added to prepare a 3% polymannuronate sample in the cholesterol solution. The mixture was mixed well and left for about 30 minutes at room temperature. Then, the mixture was centrifuged (10,000 x g for 10 min), and the supernatant was collected. The cholesterol content in the supernatant from each polymannuronate sample was measured using the well estabiiahed method described in Sperry, W. M. and M. Webb. (1950) J. Biol. Chem., Vol. 187, 97, a copy of which is attached hereto as Appendix B.

8. The measured cholesterol content in each supernatant represents the amount of the cholesterol that did not bind with the polymannuronate in each sample. From the measured cholesterol content, the cholesterol binding capability of the polymannuronate was obtained. The cholesterol bound with the polymannuronate in each sample is the initial cholesterol amount (0.1 g) less the measured cholesterol content. The cholesterol binding capability of each sample is the ratio of the cholesterol bound with the isolated polymannuronate to the initial cholesterol amount (0.1 g). Table 2 below summarizes the measured cholesterol contents in the supernatants and the cholesterol binding capabilities of the polymannuronate samples. Figure 1 following Table 2 plots the cholesterol binding capability (%) of polymannuronate versus its molecular weight.

Table 2

Sample No.	Polymannuronate Molecular Weight (KDa)	Measured Cholesterol Content (g)	Cholesterol Binding Capability (%)
1	210.3	0.065	35
2	155.5	0.063	37
3	122.1	0.063	37
4	92.6	0.060	40
5	82.9	0.036	64
6	72.3	0.035	65
7	62.8	0.031	69
8	53.2	0.031	69
9	44.2	0.038	62
10	32.4	0.055	[illegible]
11	23.0	0.056	44
12	10.2	0.062	38

Figure 1



9. As summarized and illustrated in Table 2 and Figure 1, the polymannuronate having a molecular weight of from about 40,000 Da to about 80,000 Da has up to about 197 % (69/35) of cholesterol binding capability over the polymannuronates with other molecular weights. At a minimun, the polymannuronate having a molecular weight of from about 40,000 Da to about 80,000 Da still has about 138 % (62/45) of cholesterol binding capability over the polymannuronates with other molecular weights.

Conclusion

10. From the data obtained in the above described experimentation, it is my conclusion that the polymannuronate with a molecular weight from about 40,000 Da to about 80,000 Da has a substantially higher cholesterol binding capability than polymannuronates with other molecular weight ranges. It is generally known that cholesterol bound with alginate in the human body would likely be discharged therefrom rather than remaining in the blood stream. Given the substantially higher cholesterol binding capability, it is also my conclusion that the polymannuronate with a molecular weight from about 40,000 Da to about 80,000 Da would have

substantially better cholesterol lowering or controlling ffects than polymannuronates with other molecular weight ranges.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Dated: February, 12, 2003.

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Publication

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Appendix B

A REVISION OF THE SCHOENHEIMER-SPERRY METHOD FOR CHOLESTEROL DETERMINATION

By WARREN M. SPERRY and MERRILL WEBB

(From the Departments of Biochemistry, New York State Psychiatric Institute, and the College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, May 24, 1950)

The original Schoenheimer-Sperry method for cholesterol determination (1) has been modified at several points. It is the purpose of this report to describe the revised procedure with particular emphasis on a recent investigation of the technique used for the precipitation and washing of cholesterol digitonide.

EXPERIMENTAL

Except for changes in the precipitation and washing technique, the modified Schoenheimer-Sperry method was used in the form described below for all determinations of total and free cholesterol. The blood serums studied were obtained from healthy human subjects (2); 1 or 2 ml. were extracted in a total volume of 25 or 50 ml., respectively. Aliquot portions of the extracts were precipitated by four different procedures. In Procedure A, a 0.4 per cent solution of digitonin in water was used with no other changes in the method as described below. This is the procedure which has been employed in our laboratory for some time. The solution was about 2 months old at the time the investigation was started, and about 9 months old at the end. In Procedure B, a dilute alcoholic digitonin solution, prepared as described by Sobel and Mayer (3), was used. This is the modified method, adopted as a result of this study, and described below. Procedure C was the same as Procedure B, except that precipitation was continued for only 3 hours at a temperature of 37°. In Procedure D, free cholesterol was precipitated with the dilute alcoholic digitonin solution for 3 hours at 37°, and stirring rods and the acetone-ether washing were omitted. This is the procedure recommended by Sobel and Mayer (3); it was applied exactly as they described it except that 2 ml. of extract and 1 ml. of digitonin solution were employed instead of the larger volumes used by them. All determinations were carried out in duplicate.

Results

Comparison of Aqueous and Dilute Alcoholic Digitonin Solutions—Procedures A and B were applied to twenty-five samples of serum. In one

sample the total cholesterol values were the same, and in another Procedure A gave a slightly higher result, but in the other twenty-three Procedure B yielded the larger values. In all but one of the free cholesterol determinations Procedure B also gave the higher result. Although many of the differences were small, the consistency of the results leaves no doubt that the alcoholic digitonin solution was superior to the aqueous.

The difference tended to become larger as the aqueous digitonin solution became older; in the first seventeen determinations, carried out when the solution was from 2 to 5 months old, the differences were usually less than 5 per cent. But in all but one of the last eight determinations, when the solution was 7 to 9 months old, the differences ranged from 7 to 12 per cent for total cholesterol, and from 7 to 23 per cent for free cholesterol. Comparisons with standard solutions of cholesterol confirmed the conclusion that the aqueous digitonin solution had degenerated with age.

Effect of Time of Precipitation—Procedure C was applied to eleven samples of serum, and in all cases it gave a lower value than Procedure B for both total and free cholesterol. Although most of the differences were small (1 to 9 per cent), the consistency shows that 3 hours at 37° is not adequate for complete precipitation.

Effect of Omission of Stirring Rods and Acetone-Ether Washing—Procedure D, which was proposed by Sobel and Mayer for free cholesterol determination, was applied to twenty samples of serum, and in all cases it gave a lower result than Procedure B. Although some of the differences were small, in over half they ranged from 5 to 12 per cent. In eight of the eleven instances in which both Procedures C and D were applied to the same serum, the latter gave a lower result. These findings suggest that the use of stirring rods, as described in the original method, is essential to optimal results. The omission of the acetone-ether washing might be expected to increase, rather than decrease, the values obtained for free cholesterol.

Percentage of Free in Total Cholesterol (F:T)—With the exception of slightly high values in Subject 71, all of the percentages of F:T obtained with Procedures A, B, and C were within the normal range of about 24 to 30 per cent (4). Inspection of the data, which are listed in Table I in the order in which they were obtained, suggested that, in the analyses carried out before the aqueous digitonin solution had seriously deteriorated, the variation in F:T was much less among the two or three determinations on a given individual than it was among individuals. Statistical analysis[1] of the data, except the four determinations in December, showed that this impression was correct. By the correlation technique, or by an equivalent analysis of variance, it was found that there was a highly

[1] We are indebted to Dr. John W. Fertig for the statistical analyses.

significant correlation among the values obtained by the different procedures on the same sample of serum.

Error of Method—During the investigation free cholesterol was determined in duplicate portions of serum extract 91 times, and total cholesterol 70 times. From the differences between the values obtained in pairs of determinations the error of the method was evaluated by statistical treatment[1] of the data. Within the range of concentrations encountered, the error was found to be independent of the concentration. The error also appeared to be independent of the procedure used. Hence, all of the data for each of the cholesterol fractions were treated together to give an error of measurement, represented by a standard deviation of 0.77 mg. per 100 ml. for free cholesterol, and 1.81 mg. per 100 ml. for

TABLE I

Effect of Variation in Method on Percentage of Free in Total Cholesterol

Subject No.	Date of analysis	Free in total cholesterol		
		Procedure A	Procedure B	Procedure C
		per cent	*per cent*	*per cent*
1	May 24	26.9	27.2	26.7
9	" 31	26.3	26.9	26.0
50	" 31	26.7	26.3	26.2
71	June 7	30.2	30.6	31.1
69	" 10	29.4	29.1	28.3
82	" 21	29.5	30.5	
89	" 23	25.2	24.7	24.8
70	" 23	29.2	27.4	28.5
2	" 27	28.5	29.2	28.9
60	" 28	27.0	26.3	
72	" 28	28.0	28.0	28.2
73	" 28	26.8	27.4	
77	July 5	25.0	26.1	
86	" 13	29.0	29.6	
87	" 27	25.7	26.5	25.9
8	Aug. 15	27.0	27.7	
64	" 15	27.0	26.9	
1	Oct. 5	26.9	26.8	26.9
85	" 17	27.7	27.3	
6	" 21	24.7	25.0	
84	Dec. 12	23.8	25.3	
84	" 27	24.3	27.7	
88	" 29	25.4	27.2	
60	" 29	24.2	28.4	

Each value was calculated from the averages of duplicate determinations of free and of total cholesterol.

total cholesterol. In terms of probable errors the values are, respectively, 0.52 and 1.22 mg. per 100 ml. (*cf.* (4) p. 131).

Precipitation of Cholesterol from Standard Solutions with Different Digitonin Solutions—Aqueous and dilute alcoholic solutions, freshly prepared from seven different lots of digitonin from three manufacturers, precipitated cholesterol quantitatively from two standard solutions in alcohol-acetone. All of the values were between 99 and 101 per cent recovery, except for those of 97 and 98 per cent yielded by one of the aqueous solutions.

Revised Method for Cholesterol Determination

Special Reagents—

1. Solvents. Acetone-absolute ethanol (1:1); acetone-ether (1:2); ether. We make it a practice to use only redistilled solvents and ether which is peroxide-free as tested by shaking 10 ml. with 1 ml. of 10 per cent cadmium potassium iodide solution in a glass-stoppered cylinder. The mixture is placed in a dark place for 1 hour. If any color develops, the ether should be purified by treatment with an acidified 5 per cent solution of ferrous sulfate.

2. Digitonin solution, 0.5 per cent. Dissolve 500 mg. of digitonin in 100 ml. of 50 per cent alcohol (55 ml. of 95 per cent alcohol and 45 ml. of distilled water) at 60° (3).

3. Potassium hydroxide solution made by dissolving 10 gm. of pure KOH in 20 ml. of water. If a sediment develops, the solution is filtered through sintered glass before use.

4. Acetic acid solution made by diluting 10 ml. of glacial acetic acid to 100 ml. with water.

5. Acetic anhydride, 99 to 100 per cent. The Eastman Kodak product has been uniformly satisfactory.

6. Cholesterol solutions, containing exactly 100 mg. per 100 ml., in glacial acetic acid. Working standards are made by suitable dilution of the stock solution with glacial acetic acid. The cholesterol must be anhydrous and pure. It does not keep well under ordinary conditions of storage and should be recrystallized one or more times from an anhydrous solvent and dried thoroughly in a vacuum desiccator over sulfuric acid or phosphorous pentoxide before use in the preparation of standard solutions.

Special Equipment—

1. Funnels, 2.5 cm. in diameter.

2. Filter paper, 4.5 cm. in diameter, extracted with hot alcohol, preferably in a continuous extractor (5), until it is lipide-free. The paper must be rapid, to minimize evaporation of solvent, and sufficiently retentive. Schleicher and Schüll's Sharkskin meets these requirements.

3. Stirring rods, approximately 13 cm. long.

4. Preserving jars with rubber gaskets.

5. Dropping bottles with ground-in pipettes, equipped with rubber bulbs, for the solvents.

6. Centrifuge tubes, heavy duty, 12 ml., calibrated at 2 ml., and numbered.

7. Dark cabinet, containing a water bath. A wooden packing box, equipped with a door, will serve. We use a box 18 × 10.5 inches, and 9.5 inches high. A copper pan, about 4 inches high just fits in the box. The wooden cover of the pan has thirty holes for centrifuge tubes, and provision is made for a thermometer and a funnel for adding hot or cold water as needed.

8. Photometer. We use the instrument described by Weech (6), but the method may be adapted to other instruments.

Extraction of Blood Serum—About 2 ml. of acetone-ethanol are placed in a 5 ml. volumetric flask, and 0.2 ml. of serum is added slowly in such a manner that it runs down the wall of the flask and forms a layer under the solvent. As soon as the pipette is withdrawn, the contents are mixed thoroughly by a swirling motion. A finely divided precipitate should result. The solvent is brought just to a boil on the steam bath with agitation to prevent bumping, the flask is cooled, acetone-ethanol is added to the mark, and the suspension is thoroughly mixed and filtered into a small test-tube. Aliquots of the clear filtrate for free and total cholesterol determinations are pipetted at once to avoid evaporation.

Precipitation of Free Cholesterol[2]—To 2 ml. of the filtrate in a centrifuge tube 1 drop of the acetic acid solution and 1 ml. of the digitonin solution are added. The contents are stirred thoroughly with a rod which is left in the tube. The tube is placed in a preserving jar which is covered tightly and left overnight at room temperature.

The tube is transferred to a rack, the contents are stirred gently to free precipitate which may adhere to the wall near the surface of the liquid, and the rod is removed without contact with the upper part of the tube and placed on a rack made of heavy wire and so designed that a number of rods may be held without danger of rubbing off adherent precipitate. The position of the rod is noted so that it may be returned to the proper tube. The tube is centrifuged for 15 minutes at about 2800 r.p.m., and

[2] The procedure is described for a single determination. In practice the average time per analysis may be greatly shortened by carrying through a considerable number of analyses together.

the centrifugate, which should be clear, except for the occasional presence of a few particles floating at the surface, is decanted with a slow, steady motion and watched closely in a good light as the fluid leaves the precipitate. If any is seen to be suspended, the sample is recentrifuged, or better, discarded. If this happens at all frequently, the time or speed of centrifuging should be increased. The tube is drained for a few moments and the last drop is removed by touching the lip to a clean towel. The rod is returned to the tube, the wall of the tube and the rod are washed down with 1.5 to 2 ml. of acetone-ether, the contents are stirred thoroughly, the rod is returned to the rack, the tube is centrifuged for 5 minutes, and the centrifugate is decanted. The precipitate is washed twice more in the same manner with ether. The rod is returned to the tube which may be stored for several days at this stage. If color development is to follow at once, the ether is evaporated by placing the tube for a few minutes in a moderately warm water bath.

Precipitation of Total Cholesterol—1 drop of potassium hydroxide solution is placed in a dry centrifuge tube, 1 ml. of the serum extract is added, and the mixture is stirred with a vigorous up and down motion of a rod until no droplets of the alkali solution can be seen at the tip of the tube. A preserving jar containing a layer of sand about 3 cm. deep is heated in a water bath until the temperature of the sand is 45°. The tube is placed in the sand, and the jar is covered tightly and placed in an incubator at about 38° for 30 minutes.

The tube is removed to a rack and cooled. The rod is raised, acetone-ethanol is added to the 2 ml. mark, and the alkali is neutralized to the phenolphthalein end-point with 10 per cent acetic acid. From 4 to 6 drops should be required if the drop of alkali solution was of proper size. An extra drop of acetic acid and 1 ml. of digitonin solution are added, and the sample is treated as described for free cholesterol, except that the precipitate is washed with ether only once.

Development and Reading of Color—The tubes are placed in order of reading in a sand bath at 110–115° in an oven for 30 minutes. The temperature of the water bath in the dark cabinet is adjusted to 25° and maintained there during the rest of the procedure by the addition of hot or cold water as needed. The sand bath is removed from the oven, and 1 ml. of pure glacial acetic acid is added to the first tube while it is still in the hot sand. The contents are stirred vigorously, and the tube is left in the sand while the acid is being added to the next two or three tubes; 2 or 3 minutes in all. The solution is stirred again, and the tube is removed from the sand, cooled, and placed in the water bath. The process is continued until all the tubes are in the water bath in order of reading. A tube containing 1 ml. of standard solution (0.1 mg. of cholesterol per 1 ml.) is placed at the beginning, and another at the end of the series.

An amount of acetic anhydride sufficient for the number of samples to be read is placed in a glass-stoppered flask and chilled in an ice bath. With the flask still in the ice bath, concentrated sulfuric acid is added in the proportion of 1 ml. to 20 ml. of acetic anhydride with agitation during the addition. The stopper is inserted, and the flask is removed from the bath, shaken vigorously for a few moments, and returned to the bath. A blank containing 1 ml. of acetic acid and 2 ml. of the reagent is prepared.

About 10 minutes later, when the reagent is thoroughly chilled, the first tube is removed from the 25° water bath and wiped dry, 2 ml. of the cold acetic anhydride-sulfuric acid reagent are added, the contents are stirred vigorously, the rod is removed, and the tube is returned to the bath. If the reading is to be carried out in a photometer which uses matched cuvettes of the test-tube type, the solution is poured at this point into a cuvette, which is placed in the 25° bath. The process is repeated with each tube in the series at time intervals, best controlled by a stop-watch, such that the time of color development before reading is the same for all samples. Although the color is fairly stable at its maximum from 27 to 37 minutes after addition of the reagent, it is best to read all samples after as nearly the same time of color development as possible, preferably 30 to 31 minutes after the acetic anhydride-sulfuric acid solution is added. The temperature of the water bath is noted, and adjusted, if necessary, at frequent intervals during the color development and reading.

The process of reading is not described, since it will vary somewhat with the instrument used. The foregoing procedure yields sufficient solution for the instrument we employ (6), and for some others, but some of the commonly used photometers require a larger volume. The method may be adapted to such instruments by suitable adjustment of the volumes. In general, it may be varied in the following ways to meet special conditions: (1) The proportion of serum volume to extract volume may be decreased to compensate for exceptionally high cholesterol concentrations, but not increased. (2) The volume of extract in which precipitation is carried out may be varied at will, provided that 1 drop of alkali solution per 1 ml. of extract is added in the determination of total cholesterol, and that 1 ml. of digitonin solution is added for each 2 ml. of acetone-ethanol. (3) The volume in which color is developed may be varied without reference to the volume in which precipitation was carried out, provided that 2 ml. of the acetic anhydride-sulfuric acid reagent are added for each 1 ml. of acetic acid.

DISCUSSION

Aqueous solutions of digitonin, necessary under the conditions of the original Schoenheimer-Sperry method, have not been entirely satisfactory, because precipitates form in them on standing to a degree which varies widely among different lots of digitonin from different manufacturers. The present study revealed a far more serious objection to aqueous digitonin solutions. It is evident from the results that such solutions, although usually reliable when freshly prepared, cannot be depended on to keep for long periods of time. This difficulty was not encountered with the Hoffmann-La Roche digitonin, which was used exclusively until the supply was cut off by the war, and we have the strong impression, based on much experience, that aqueous solutions of that product did not deteriorate with age, but it must be confessed that no systematic tests, such as that described here, were carried out. Modifications of the original method, particularly the use of acetic instead of hydrochloric acid in neutralizing the alkali in total cholesterol determinations, have obviated the requirement of an aqueous solution, and the findings of this investigation show that the Sobel-Mayer dilute alcoholic solution is preferable, if used under the conditions described above. It did not give complete precipitation under the conditions proposed by Sobel and Mayer (3).

The finding of a close agreement among the F:T values yielded by Procedures A, B, and C (Table I) is of considerable interest in its bearing on the mechanism of precipitation of cholesterol digitonide. Since the amount of total cholesterol present is about twice that of free cholesterol, whereas the quantity of digitonin and total volume are the same in both samples, the result suggests that the rate of precipitation is independent of the amount of cholesterol, at least within the range of quantities encountered in this study, and that for an unknown reason the rate at which the aqueous digitonin solution, used in Procedure A, was able to precipitate cholesterol was reduced.

The F:T results are also of interest in another connection. The discovery (4) that F:T is maintained within a narrow range of variation in healthy persons has been confirmed by a large number of determinations in this laboratory and by other investigators (7). The proportion is maintained within the same narrow range in all pathological conditions we have studied, except liver disease and acute infection. Because the range of variation is small, little attention has been given to the possible significance of variations among individuals within the range. The statistical analysis of the data of this study shows that the variations in F:T among subjects were real and not due to some chance event. This conclusion was confirmed by a study of the duplicate determinations obtained with Procedure B. Each of the two free cholesterol values was paired at random with one of the total cholesterol concentrations and the F:T values were calculated. These are plotted arbitrarily against each other in Fig. 1. The high correlation, indicated by the chart, was borne out by statistical analysis of the data, and it may be concluded that variations in F:T among individuals within the normal range are to only a small extent the result of experimental error.[3] The factors which control F:T in each individual remain to be determined.

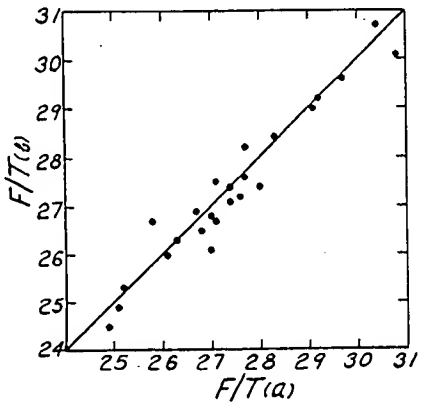

FIG. 1. A comparison of duplicate determinations of the percentage of free in total cholesterol (F:T). One of the duplicates (*a*), selected at random, is plotted against the other (*b*).

SUMMARY

A study of the precipitation of cholesterol by digitonin under various conditions within the framework of a revised Schoenheimer-Sperry method gave the following results: (1) Precipitation for 16 to 18 hours at room temperature with solutions of digitonin in approximately 50 per cent alcohol, or with freshly prepared aqueous solutions, yielded maximal values. (2) Aqueous digitonin solutions may lose their ability to precipitate cholesterol quantitatively as they become older. (3) Precipitation is not complete in 3 hours at 37°. (4) The rate of precipitation appears to be independent of the amount of cholesterol present within the range of quantities encountered in this study.

A revision of the Schoenheimer-Sperry method is described.

Variations in the percentage of free in total cholesterol among healthy

[3] This conclusion ignores the remote possibility that the extraction of one or the other cholesterol fractions may be incomplete to a variable degree.

persons within the normal range are much larger than can be explained by the error of the determination.

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THE EFFECT OF INCREASING AGE ON SERUM CHOLESTEROL CONCENTRATION

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Determinations of the total cholesterol concentration of the blood serum in twenty-five healthy subjects on two or more occasions over periods of time up to 28 months in length led to the conclusion that the variability of this constituent is much less within a given individual than it is among individuals, and the hypothesis was advanced that in each healthy person the serum cholesterol concentration is maintained, perhaps throughout life, at a constitutional level, from which large deviations do not occur in the absence of unusual stress (1). Other investigators (2–4) have reported findings in general agreement with this hypothesis, and strong support for it was furnished by the finding (5) that the average cholesterol concentrations in children, calculated for each year of age, did not change appreciably from the 2nd month to the 13th year of life and were almost the same as the average found in healthy adults between the ages of 19 and 43 years.

Recently Keys (6) found a considerable increase in the average serum cholesterol concentration of healthy men with increasing age, from 173 mg. per 100 ml. at 19 years to 252 mg. at 52.5 years of age, and Gram and Leverton (7) reported a similar result in women. These findings are inconsistent with the hypothesis stated above.

Because of this apparent discrepancy it seemed worth while to ascertain whether the serum cholesterol level had increased in the individual subjects of the previous study (1) during the 13 to 15 years since that investigation was carried out.

EXPERIMENTAL

During 1949 the cholesterol concentration of the blood serum was determined in twenty-two of the twenty-five subjects of the earlier investigation (1).[1] In two (Subjects 1 and 60) the determination was repeated, and in one (Subject 50) an analysis had been carried out in 1942. The methods are described in the preceding paper (8).

[1] We are deeply indebted to our colleagues who cheerfully furnished samples of their blood; particularly to the ten who participated in the investigation by preparing extracts and mailing them to us.